Evaluation the resistance of some cucumber cultivars to root-knot nematode *(Meloidogyne incognita and M. javanica)*

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ABSTRACT---The root-knot nematode, caused by Meloidogyne spp., is one of the major limiting factors affecting plant growth and yield causing an estimated $100 billion loss per year worldwide. In the present study, six cucumber cultivars were evaluated for their resistance against M. incognita and M.javanica under greenhouse conditions. The nematode caused great reduction to the growth parameters.

None of the tested cultivars was found completely resistant to the infection root-rot nematode. Eshrak cucumber cultivar was the highest susceptible one (>100 galls were recorded on the roots) and showed the maximum reduction in the growth among the evaluated cultivars. Both Hesham and Bet alfa F1 cvs. were susceptible (71-100 galls were recorded on the roots). Meanwhile, cv. Bet alpha and the two Wafer and Nimes hybrids were moderately susceptible (31-70 galls were recorded on the roots) with less reduction in the growth in addition to less damage by the nematode as compared to the susceptible cvs. The tested mineral salts led to decreasing root galls as well as nematode reproduction on cucumber roots with an increased to the growth of cucumber plants and some chemical contents and phenolic compounds in the healthy and infected plants.

Extracts of un-sprayed leaves showed 11 bands with molecular weights ranging between 35-109 KD. The sprayed leaves with the tested salts resulted in an increase in the number of separated bands with molecular weights ranging between 17 and 118 KD compared to the control (35-109 KD).

Keywords: Cucumber , cultivars , root-knot nematodes, mineral salts, sugars, phenols , amino acids , protein , Electrophoretic studies.

1 INTRODUCTION

Cucumber (*Cucumis sativus* L.) is one of the most important vegetable crops grown in Egypt for local consumption and exportation.The cultivated area reached about 22676.19 feddan, of which more than 50 % of this area is under protected cultivation. Increasing plant productivity and improving uptake and quality are important. Cucumber is highly susceptible to *Meloidogyne* spp. and considerable plant damage and yield loss occurs worldwide. In many cases nematodes do not affect yields directly, but bacterial and fungal infections may occur in root lesions caused by nematodes. Synthetic pesticides, though instantaneously effective, are usually prohibitively expensive, not readily available, may cause hazards to both human and livestock, and inflict injury to the environment.

Notable among the alternatives to nematicides is the use of resistant cultivars, which are inexpensive and eco-friendly. Resistance to pathogen infection can be induced in plants by a wide range of biotic and abiotic agents (da Rocha and Hammerschmidt, 2005 and Lyon, 2007). It has been reported that some phosphate salts could induce local and systemic resistance to various plant diseases of cucumber (Mucharromah and Kuc, 1991). In trials to explain the effect of physiological and chemical changes following salt (s) application . Bourlarye *et al*. (2005) reported that biochemical analyses showed similar levels of 4-coumarate: CoA ligase (4 CL), protein accumulation for all treatments . However, the results support the idea that induced resistance in cucumber is largely correlated with rapid de novo biosynthesis of flavonoid phytoalexin compounds.

This research objective to evaluate the effect of some mineral salts on reproduction of *M. incognita* and *M. javanica* on cucumber and some chemical institutes in the healthy and infected plants.

2 MATERIAL AND METHODS

Evaluation of six cultigens

A greenhouse study was conducted to show how well reproduction factor ($R = P_f / P_i$) along with gall index could determine resistance in species of *Cucumis* . The experiment was arranged in a randomized complete block design with four replications. A treatment combination consisted of one *Cucumis* cultigen and one root-knot nematode species in a 25 cm diameter (1500 cm^3 volume) pot. Pots contained sterilized sand and clay in a 1:1 ratio. Three cucumber seeds of any of the four cultivars , *i.e.* Eshrak-28, Hesham , Bet alpha F_1 , Bet alpha and the two hybrids of Wafer and Nimes of *C. sativus* were sown in each pot and thinned into two seedlings, 10 days after sowing . Each pot was inoculated two weeks after planting with 2000 root-knot nematode fresh hatched juveniles of any of the two root-knot nematodes ,*i.e. M. incognita* and *M. javanica* using a technique developed by Hussey and Barker (1973). Four post were used for each treatment and four pots for each

cultigen were kept without inoculation and served as a control. Plants were rated 8 weeks after inoculation (10 weeks after planting) for root-knot nematode damage using a gall index (0 to 100% of roots galled) and then measuring the number of eggs on roots. The number of eggs on the roots of each plant was determined using the technique for obtaining root-knot nematode eggs as described by Hussey and Barker (1973). The fresh weight of shoots and roots were recorded.

Effect of soaking seeds of cv. Eshrak-28 in some mineral salts on reproduction of *M. javanica* and growth of cucumber plants in the greenhouse:

Seeds of cv. Eshrak-28 were soaked overnight in 10 mg L^{-1} of different salts (Na_2HPO_4, K_2HPO_4, ascorbic acid, salicylic acid, citric acid and indol acetic acid) . Each treatment was replicated three times with three pots planted with seeds soaked in water to serve as control. Each plastic pot, 20-cm-diam., was filled with autoclaved sandy loam soil, about 3 kg, and planted with five seeds of sunflower, hybrid aroflower and thinned into three seedlings two weeks after germination . Each pot was infested with 1,500 second-stage juveniles. Pots were randomly arranged in a greenhouse bench and watered as needed. Sixty days after soil infestation, the experiment was terminated and dry root and shoot weights, number of galls per root system and total count of nematodes per pot were recorded for each treatment. Also, the reproductive factor (Rf), which is the result of dividing the final count of nematode by the initial number used in soil infestation.

Chemical analyses:

Preparation of root extracts:

A representative samples ,each of one gram, were obtained from fresh roots of all treatments at the end of the experiment. Such samples were cut into small pieces and immediately plunged into 95% boiling ethanol for 10 minutes to kill the tissues. The extraction was then resumed in a Soxhelt apparatus using 75% ethyl alcohol as an extractant until the perorate was colorless, (about 8-10 hrs). Alcoholic extractions were filtrated and evaporated to near dryness on mild water bath, 60ºC. The dried residue was re-dissolved in 5 ml of 50% isopropanol. These extracts were then used for determination of sugars, phenols and amino acids contents as follows:

Determination of sugars:

Total and reducing sugars were determined spectrophotometerically by picric acid method described by Thomas and Dutcher (1924). The sugar contents were calculated as mg glucose/ gm fresh weight of roots. Values were obtained from a standard curve prepared for glucose. The color density was recorded by using spectrophotometer in the presence of a blank at wave length of 540 nm.

Determination of phenolic compounds:

Phenolic compounds were determined using the colorimetric method of Folin-Denis reagent described by Snell and Snell (1953). The color intensity was recorded using spectrophotometer in the presence of a blank (containing all reagents without the extracts) at the wave length of 560 nm. The concentrations of free and total phenols were calculated as mg caticol / g fresh weigh. Values were obtained from standard curve constructed for caticol in an identical way.

Determination of The total amino acids:

The total free amino acids were determined as mg leucine / g fresh weight by modified colorimetric ninhydrin method that was used by Rason (1959). The color density was immediately recorded using spectrophotometer in the presence of a blank at the wavelength 570 nm. Values were obtained from standard curve prepared for leucine.

Electrophoretic studies:

Total protein extraction:

Sodium dodecyle sulphate, polyacrylamide gel electrophoresis (SDS-PAGE) technique was performed for separating proteins by the method described by Laemmili (1970), and modified according to Hames (1995). The modification, was reduced TEMED from 30 µl to 25 µl and also APS was reduced from 1.5 ml to 1.3 ml. Approximately 5 g plants at the 3-4 leaves growth stage were sampled for protein analysis was ground in a mortar and pestle in liquid nitrogen. Crushing continued until the sample completely homogenized. The crushed samples were transferred to 1 ml Eppendorf tube brought to 200 µl with extraction buffer (50 m M tris-HCl buffer, pH 6.8, glycerol 10% w/v, ascorbic acid 0.1%, cycteine hydrochloride 0.1 w/v). Centrifugation, 18,000 rpm for about 30 min, was carried out to remove debris. The protein content in supernatant was estimated according to the method of Bradford (1976) by using bovine serum albumin as a standard protein. Protein content was adjusted to 2 mg / ml per sample.

Stock solution:

1. Separating gel buffer (1.5 M tris-HCl, pH 8.8): Absolutely 18.17 g of tris-base was dissolved in 50 ml deionized water, and pH was adjusted to be 8.8 using concentrated HCl. The final volume was made up to 100 ml. with distilled water.
2. Staking gel buffer (0.5 M tris-HCl, pH 6.8): Tris-base (6.05 g) was dissolved in 50 ml deionized water and pH was adjust to 6.8 with concentrated HCl. The final volume was made up to 100 ml. with distilled water.
3. Sodium dodecyl sulphate solution (10 % w/v SDS): Sodium dodecyl sulphate (2.5 g) was dissolved in 25 ml deionized water and heat at 60 °C to facilitate dissolving.
4. Ammonium per sulphate solution (1.5 w/v APS): Ammonium per sulphate (APS) 0.15 g was dissolved

in 10 ml deionized water and kept at 4ºC. The solution is unstable and must be made just before use.

5. Electrophoresis buffer (pH 8.3-8.5): The tank buffer consists of three g tris-base, 14.4 g glycine and one g sodium dodecyl sulphate dissolved in 1000 ml deionized water.
6. Monomer solution (Acrylamide stock solution): Acrylamide (29.2 g) and tris, N.N-methylene bis acrylamide (Bis) 0.8 g were dissolved in a final volume of 100 ml of deionized water, any insoluble materials were removed by filtration through Whatman filter paper No.1.

Preparation of gels:

Preparation of gels was made as described by Laemmili (1970).

1. Resolving (separating) gradient gel: Gel mixture for polyacrylamide gel electrophoresis 11% (SDS-PAGE) is prepared as follows:

Acrylamide solution 30 %	10 ml
1.5 M tris-HCl (pH 8.8)	7.5 ml
10 % (w/v) SDS	0.3 ml
Deionized water	11.0 ml
1.5 % (w/v) APS	1.5 ml
TEMED	15.0 µl

2. Staking gel:

Acrylamide solution 30%	3.5 ml
0.5 M tris-HCl (pH 6.8)	7.5 ml
10 %(w/v) SDS	0.3 ml
Deionized water	17.8 ml
1.5 % (w/v) APS	1.5 ml
TEMED	30 µl

3. Preparation of samples:

Sodium dodecyl sulphate (SDS) was added to the sample at a rate of 4 mg SDS/1 mg protein, then 50 µl 2-mercaptoethanol were applied to each 950 ml of the sample, then the mixture was heated at 100ºC in water bath for 3-5 min.

4. Pouring the separating and staking gel: The resolving gel was poured between glass sandwich using scientific instruments (San Francisco CA, USA, Model XPO77 Hoefer) and gently covered with 1 cm of water. Polymerization started within 25-30 min. after pouring. The stacking gel was then poured and allowed for polymerization after about 30 min.
5. Loading of the samples: Twenty micro-liters of this crude protein solution were applied to the wells of the stacking gel. The samples were covered with electrode buffer. Few drops of bromophenol blue (4 mg/100 ml deionized water) were added to the electrode (tracking dye).
6. Gel running: Electrophoresis was performed in a vertical slab mold (Hoefer Scientific Instruments, San Francisco, CA, USA, model LKB 2001, measuring 16 × (18 × 0.15 cm). Electrophoresis was carried out at 30 milliamper (m.A.) at 10ºC for 3 hours.
7. Staining the gel with silver nitrate: The silver staining method for protein described by Sammons *et al.* (1981) was used. This method of staining is sensitive and detect as little as 2 ng of protein in a single band.

Table (1). Silver staining method for proteins (Sammons *et al.*, 1981).

Step.	Staining procedure.	Time
1	Fix gels in 50% ethanol containing 10% acetic acid.	3 × 1hr
2	First wash in 50% aqueous ethanol containing 10% acetic acid.	2hrs.
3	Two washes in 25% aqueous ethanol containing 10% acetic acid.	2 × 1hr
4	Two washes in 10% aqueous ethanol containing 0.5% acetic acid.	2 × 1hr
5	Equilibrate gel in a degassed aqueous solution of silver nitrate (1.9 g/ l) the volume of solution being about 3 times as the volume of gel.	2hrs
6	Rinse briefly in degassed water.	-
7	Place in reducing bath consisting of 0.75 M NaOH 87 mg/l of NaOH and 15 ml of 37% formaldehyde which is added to the solution immediately before use. The volume used should be 5.5 times the gel volume.	10 min.
8	Place in color enhancing solution (5.5 times gel volume) consisting of 7.5 g/l sodium nitrate in water.	1hr
9	Transfer into fresh sodium carbonate (7.5 g/ l water).	1hr
10	Transfer into sodium carbonate (7.5 g/ l).	1hr

Statistical analysis

The data obtained were statistically analyzed according to Snedecor and Cochran (1967). Averages were compared at 0.05 level of probability using least significant difference (L.S.D) as mentioned by Fisher (1948).

3 RESULTS AND DISCUSSION

Reaction of some *Cucumis* cultigens to root-knot nematode:

All *Cucumis* cultigens evaluated, *i.e. i.e.* Eshrak-28, Hesham , Bet alpha F_1 , Bet alpha and the two hybrids of Wafer and Nimes were of little difference in root galling.

Selected cultigens evaluated, are listed in Table (2). The complete data set will be entered into the six *C. sativus* cultigens. Eshrak-28, Hesham , Bet alpha F_1, Bet alpha, Wafer hybrid and Nimes hybrid were evaluated to *M. incognita and M.javanica.*. Zhong and Bie (2007) mentioned that both nematode species (*M. javanica* and *M. incognita*) reduced cucumber fruit yield, which was more evident when *M. javanica* was inoculated. Grafted and un-grafted plants were susceptible, because they enabled the multiplication of nematodes, with a reproduction factor, at 72 days after inoculation, ranging from 3.57 to 15.04 with the highest value in cucumber un-grafted and inoculated with *M. javanica* (Salata, *et al.*, 2012) .

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Table 2. Reproduction factors (Rf) and gall indices (gall) for 6 cultigens of *Cucumis* spp. infected with 2 species of root-knot nematode.

Cultigen	*M. incognita*		*M. javanica*	
	Rf	Gall	Rf	Gall
Eshrak-28	12.6	198.0	15.0	194.0
Hesham	4.5	76.0	1.4	65.0
Bet alpha F_1	2.3	52.0	1.0	45.0
Bet alpha	4.5	59.0	1.3	57.0
Wafer hybrid	4.3	64.0	0.4	35.0
Nimes hybrid	0.5	39.0	0.7	45.0
LSD at 5%	0.1	2.0	0.1	2.0

Table 3. Effect of *Cucumis* cultigens on the parameters of 2 species of root-knot nematode .

Cultigen	*M. incognita*		*M. javanica*	
	Eggs /5 g roots	Juveniles /250 g. soil	Eggs /5 g. roots	Juveniles /250 g. soil
Eshrak 28	1900	3000	2000	3500
Hesham	1000	800	1100	650
Bet alpha F_1	60	400	1400	300
Bet alpha	800	150	1300	400
Wafer hybrid	300	60	250	35
Nimes hybrid	20	110	32	150
LSD at 5%	450	88	14	450

Results of the effect of *Cucumis* cultigens on the parameters of nematode are presented in Table (2). The results show significant differences among cultigens.

Data shown in Table (3) indicate that the effect of different mineral salt treatments on reproduction factor of *M. javanica* was differed according to the treatments.

In the greenhouse experiment, all the used minerals increased shoot weight, while their effect on root weight was fluctuated compared to the un-treated control. The increase of these two growth parameters may be due to these treatments are growth promoters (indol acetic acid) or sources for nutritional elements such as phosphorus, potassium and sodium. Ascorbic, salicylic and citric acids may have direct or indirect role in this respect. Most of the tested minerals reduced reproduction of *M. javanica* on cucumber proving their potentiality in controlling this serious pest compared with untreated control, especially in K_2HPO_4 and citric acid, which recorded 0.0 Rf then Indol acetic acid, which recorded 0.28 Rf . This effect against *M. javanica* could be attributed to the role of the used compounds in induction of plant resistance as reported by Zinovieva *et al.* (1995 and 1998). Since some of the used chemicals are growth regulators they may enhance some metabolic cycles and pathways that cause accumulation of some metabolites that resist pathogenic organisms. The effect of the tested chemicals on the phenolic, sugars and total amino acids was determined. Regarding to sugars content, the obtained data show a positive correlation between the amounts of total or reducing sugars and the used compounds (Table,5). Amounts of total sugars ranged from 2.036 mg/g for plants treated with Na_2HPO_4 to 4.04 mg/g for plants treated with citric acid, while amounts of reducing sugars were ranged between 0.317 to 0.415 mg/g for plants treated with K_2HPO_4 or Na_2HPO_4, respectively.

Also, the amounts of total phenols were increased by the used mineral salts. The highest amount was for K_2HPO_4 treatment, being 16.553mg/g plant. The amounts of free phenols show certain trend with regard to the used compounds .The highest amount was produced by K_2HPO_4 treatment, being 12.753 mg / g fresh weight. Meanwhile, ascorbic acid reduced free phenols to 3.9 mg/g fresh weight. The highest amount of total amino acids was occurred with citric acid followed by K_2HPO_4 and salicylic acid, while it was 0.007 mg/g fresh weight for the non-treated plants .

Most of the tested chemicals reduced reproduction of the *M. javanica* on cucumber , proving their potentiality in controlling this serious pest. Thr obtained data are in agreement with those reported by Zinovieva *et al.* (1995 and 1998), where the used chemicals may be enhance some metabolic cycles and pathways that cause accumulation of some metabolites and / or induce acquired resistance that resist or tolerate the pathogenic organisms. The increase in sugars by the used chemicals could be due to enhancing the metabolism and accumulation of metabolites that contain sugars. Another possibility is that the higher nematode suppression and

consequently less consumption of nutrient including sugars.

Table 4. Effect of certain mineral salts on the reproduction of *M. javanica* and growth of cucumber plants.

Treatments	**Shoot Wt.(g)**	**Root Wt. (g)**	**No. of galls per root system**	**Rf**
Na_2HPO_4	9.67	4.33	63.67	1.12
K_2HPO_4	11.67	5.67	0.00	0.00
Ascorbic acid	13.00	5.67	244.00	1.88
Salicylic acid	9.00	4.67	119.00	1.44
Citric acid	16.33	6.00	0.00	0.00
Indol acetic acid	19.33	8.00	23.33	0.28
Non-treated (control)	5.00	4.00	211.00	1.94
LSD 5%	0.307	0.098	17.87	0.002

The increase in the free phenols by the applied

To found the biochemical differences among the treated infected cucumber cultivars , protein was extracted as described under " total protein" and electrophoresed on one dimension Sodium dodecyle sulphate, polyacrylamide gel electrophoresis (SDS-PAGE) was carried out. Plants at the 3-4 leaves growth stage were sampled for protein analysis (Table, 6). Leaf extracts were subjected to electrophoresis for 6 hours. Extracts of non-sprayed leaves showed 11 bands with molecular weights ranging between (35-109 KD) .The sprayed leaves with the salts in concern increased the number of separated bands with molecular weights ranging between 17 and 118 KD, compared to the control (35-109 KD).

The decrease in the total amino acids by the used compounds could be attributed to lower the infection and consequently lower replenish of proteins from the plant cell that adjacent to the infected ones (Nandi *et al.*,2003)

Our results give an approach to control *M. javanica* in cucumber using compounds that are safer than nematicides. These results should be considered when designing an integrated pest management program for root-knot nematodes or other nematode pathogens in cucumbers and other crops.

Table 5. Effect of certain mineral salts, on some chemical analysis of Eshrak-28 cucumber cv.

Treatment	Concentration (mg/g fresh weight)				
	Total sugars	Reducing sugars	Total phenols	Free phenols	Total amino acids
Na_2HPO_4	2.036	0.415	8.610	4.110	0.068
K_2HPO_4	2.595	0.317	16.553	12.753	0.155
Ascorbic	2.127	0.405	9.15	3.900	0.075
Salicylic	2.214	0.413	8.756	6.370	0.113
Citric	4.040	0.365	9.727	9.008	0.171
IAA	2.151	0.405	10.04	7.426	0.037
Control	1.516	0.325	11.67	3.890	0.007

Table 6. Electrophoritic analysis of proteins in cucumber treated with certain mineral salts.

Protein marker M.W. (kd)	Na_2HPO_4 M.W. (kd)	K2HPO4 M.W. (kd)	Ascorbic acid M.W. (kd)	Salicylic acid M.W. (kd)	Citric acid M.W. (kd)	Indol acetic acid M.W. (kd)	Control M.W. (kd)
66.00	64.0	118	102	82	92	113	109
45.00	54.0	102	93	71	82	102	91
25.00	46.0	91	83	69	71	91	82
18.00	42.0	79	71	65	65	71	71
---	39.0	65	68	59	59	65	65
---	36.0	49	64	45	43	58	59
---	34.0	33	35	43	40	33	55
---	---	26	---	36	31	26	49
---	---	23	---	34	21	17	45
---	---	21	---	26	20	--	42
---	---	19	---	21	17	---	35

To find the biochemical differences between the treated infected cucumber plants prepared as described under " total protein " were extracted and electrophoresed on one

dimension Sodium dodecyle sulphate, polyacrylamide gel electrophoresis (SDS-PAGE). Plants at the 3-4 leaves growth

stage were sampled for protein analysis (Table, 5). Composite leaves extracts were subjected to electrophoresis for 6 hours. Extracts of non-sprayed leaves showed 11 bands with molecular weights ranging between (35-109 KD). The sprayed leaves with the salts in concern increased the number of separated bands with molecular weights ranging between 17 and 118 KD compared to the control (35-109 KD).

Induced Systemic Resistance (ISR) of plants against pathogens is a widespread phenomenon that has been intensively investigated with respect to the underlying signaling pathways as well as to its potential use in plant protection.

Mukherjee *et al.* (2012) showed that shoot weight of infected plants with nematodes and treated with SA were increased compared with infected plants without treatment, they also showed that numbers of root galls and eggs/g root decreased when plants were treated with salicylic acid(SA). In this study, SA has been tested as an inducer of resistance against *M. javanica*. It was proved that various stages of nematode infestation process, i.e. penetration, establishment, development into gravid females and reproduction, may be act as inhibition. Hari *et al.* (2012) concluded that SA activated glutathione metabolism, imparted resistance against *M. incognita* and improved yield and fruit quality. They indicated a link between SA and activation of glutathione metabolism during a compatible plant-nematode interaction to cause sulfur-induced resistance besides SA-induced defense enhancing yield and functional food quality of tomato in terms of elevated GSH level in fruit. Also, Hajra (2015) mentioned that proline and other biochemical parameters effect were evaluated on bottle gourd (*Luffa cylindrica* (L.) Roem) in control, mycorrhizal and other biotic and abiotic soil components treated plants. He found that high proline concentrations reflected on the leaves of treated plants as compared with mycorrhizal treated plants. The decrease in shoot length, number of leaves and leaf area were observed in non- mycorrhizal and plant treated with other biotic and abiotic stresses. High amount of proline was produced in non-mycorrhizal and stressed plants in result of reduced availability of water and dry matter translocation to the shoots. The proline contents of Luffa leaves showed a vast difference in different treatments. It may be concluded here that proline is an indicator of environmental stresses imposed on plants.

Our results give an approach to control *M. javanica* in cucumber using compounds that are safer than nematicides. These results should be considered when designing an integrated pest management program for root-knot nematodes or other nematode pathogens in cucumber and other crops.

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